ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit

Protocol

Applied Biosystems

For Research Use Only. Not for use in diagnostic procedures.

Information in this document is subject to change without notice. Applied Biosystems assumes no responsibility for any errors that may appear in this document. This document is believed to be complete and accurate at the time of publication. In no event shall Applied Biosystems be liable for incidental, special, multiple, or consequential damages in connection with or arising from the use of this document.

Notice to Purchaser: Limited License

A license under the process claims of U.S. patents or their foreign counterpart claims, has an up-front fee component and a running-royalty component. The purchase price of ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit includes limited, non-transferable rights under rights under the running-royalty component to use only this amount of the product to practice the DNA sequence and fragment analysis processes described in said patents when this product is used in conjunction with an Authorized DNA sequence analysis instrument whose use is covered under the up-front fee component of these patents. No other rights are granted expressly, by implication, or by estoppel, or under any other patent rights owned or licensable by Applied Biosystems. Further information relating to the purchase of licenses for DNA sequence and fragment analysis and other applications may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404, U.S.A.

Notice to Purchaser: Limited License

The purchase of the ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit includes a limited, nontransferable, non-exclusive license (without the right to resell, repackage, or sublicense) under U.S. patents, corresponding foreign counterparts and patent applications, to use this product solely with an Applied Biosystems commercial automated DNA sequencing machine or other authorized automated DNA sequencing machines that have been authorized under these patents by Applied Biosystems. No license is hereby granted for the use of this kit or the reagents therein, in any other automated sequencing machine. Such license is granted solely for research and other uses that are not unlawful. No other license is granted expressly, impliedly, or by estoppel. For information concerning the availability of additional licenses to practice the patented methodologies, contact: Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404. Patents are pending in countries outside the United States.

Notice to Purchaser About Limited License

This kit is sold pursuant to a limited sublicense from Amersham International plc under one or more U.S. Patent Nos. 5,498,523; 5,614,365 and corresponding foreign patents and patent applications. The purchase of this kit (reagent) includes a limited non-exclusive sublicense (without the right to resell, repackage or further sublicense) under such patent rights to use this reagent for DNA sequencing or fragment length analysis solely with an Applied Biosystems commercial automated sequencing machine or other authorized DNA sequencing machines that have been authorized for such use by Applied Biosystems, or for manual DNA sequencing. No license is hereby granted for use of this kit, or the reagents therein, in any other automated sequencing machine. Such sublicense is granted solely for research or other uses that are not unlawful. No other license is granted expressly, impliedly, or by estoppel. For information concerning the availability of additional license to practice the patented methodologies, contact: Amersham Life Science, Inc., Vice President, Regulatory Affairs, P.O. Box 22400, Cleveland, Ohio 44122. Patents are pending in countries outside the United States.

Notice to Purchaser: Limited License

The purchase price of the ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit includes a limited, nontransferable license under U.S. patents or its foreign counterparts, to use only this amount of the product for DNA Sequencing and related processes described in said patent solely for the research and development activities of the purchaser. No license under these patents to use the PCR Process is conveyed expressly or by implication to the purchaser by the purchase of this product. A license to use the PCR Process for certain research and development activities accompanies the purchase of certain reagents from licensed suppliers such as Applied Biosystems when used in conjunction with an Authorized Thermal Cycler, or is available from Applied Biosystems. Further information on purchasing licenses to practice the PCR Process may be obtained by contacting the Director of Licensing at Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404.

Printed in the USA, 06/2010
Part Number 4390038 Rev D

Contents

1 Introduction

2 *Preparing the Templates*

3 New Cycle Sequencing Protocols

4 Purifying Extension Products

5 Sample Electrophoresis

A Selecting Sequencing Primers

B Control DNA Sequence

C Technical Support

1 Introduction

Chapter Summary

In This Chapter The following topics are covered in this chapter:

About the Kit

Reagent Requirements

- The ABI PRISM® dGTP BigDye™ Terminator v3.0 and the ABI PRISM® BigDye™ v3.0 Ready Reaction Cycle Sequencing Kit with AmpliTaq® DNA Polymerase, FS, requires unique instrument (matrix) files for the ABI PRISM® 377 DNA Sequencer and ABI PRISM® 373 DNA Sequencers with the ABI PRISM® BigDye™ Filter Wheel installed. The instrument file created for the BigDye chemistry v1.0, v2.0, and dRhodamine chemistry is not appropriate for use with the dGTP v3.0 chemistry.
  - The 377 and 373 instruments require the ABI PRISM® BigDye™ Matrix Standards v3.0 (P/N 4390421) for instrument (matrix) file generation.
- The dRhodamine Matrix Standards and Matrix Standard Set DS-01 are not compatible with dGTP BigDye™ terminators v3.0, BigDye™ terminators v3.0, or BigDye™ primers v3.0.
- There are new v3.0 mobility files for all existing platforms.
- The basecallers are the same.

dGTP BigDye Terminator v3.0 Kit

We have developed the ABI PRISM dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit for use with difficult templates where the standard terminator kits give data with early signal loss.

This kit uses dGTP in the deoxynucleoside triphosphate mix instead of the dITP used in standard ABI PRISM dye terminator cycle sequencing kits. The dITP is used in dye terminator kits to minimize band compressions. However, this substitution can lead to early signal loss in some sequence data.

IMPORTANT Because of compressions (see page 1-6), we do not recommend using the dGTP BigDye Terminator v3.0 Kit for routine sequencing. It should be used only where the standard terminator kits do not give good data.

Kit Format

The kit combines AmpliTaq® DNA Polymerase, FS, the BigDye terminators v3.0, and the following required components for the sequencing reaction.

The following items are premixed into a single tube of Ready Reaction Mix:

- BigDye terminators v3.0 (dATP, dCTP, dGTP, and dUTP)
- AmpliTaq DNA Polymerase, FS
- r*Tth* DNA polymerase
- Magnesium chloride
- Buffer

The user provides the needed templates and primers.

Cycle Sequencing with AmpliTaq DNA Polymerase, FS

This kit formulation contains the sequencing enzyme AmpliTaq DNA Polymerase, FS. This enzyme is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site. This results in less discrimination against dideoxynucleotides, which leads to a much more even peak intensity pattern.

This enzyme also has a second mutation in the amino terminal domain that virtually eliminates the 5´→3´ nuclease activity of AmpliTaq DNA Polymerase. The enzyme has been formulated with a thermally stable inorganic pyrophosphatase to eliminate problems associated with pyrophosphorolysis.

Cycle sequencing protocols that rely on the use of AmpliTaq DNA Polymerase, FS offer the following advantages over traditional sequencing methods:

- Less hands-on operation
- No alkaline denaturation step required for double-stranded DNA
- Same protocol for both single- and double-stranded templates
- Less starting template needed
- More reproducible results

dGTP BigDye Terminator v3.0 Appearance on the 377 or 373 Instrument Gel Image

The dye/base relationships and colors of the dGTP BigDye terminators v3.0 as they appear on the gel image are shown below for the 377 and 373 instruments.

Base	Terminator	Color of Bands on ABI PRISM 377 or 373 Instrument Gel Image
A	V3 Dye 2	Green
C	V3 Dye 4	Red
G	V3 Dye 1	Blue
T	V3 Dye 3	Yellow

Difficult Templates

When problems are encountered with a particular template, use the table below to choose the best approach. This table shows which types of difficult templates should be sequenced with the dGTP BigDye Terminator v3.0 Kit and which are best sequenced with the ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit using altered reaction conditions.

Note Sequence contexts refer to the extension strand, *i.e.*, the sequence that is seen in the electropherogram.

Template Characteristic at or Near the Stop Region	Chemistry to Use
GT-rich	dGTP BigDye terminator v3.0
G-rich	dGTP BigDye terminator v3.0
GC-rich	BigDye terminator v3.0: ♦ Alter reaction conditions to improve template denaturation (refer to pages 7-32 and 7-33 of the *Automated DNA Sequencing Chemistry Guide* for methods). ♦ If the sequence data does not improve with the BigDye Terminator v3.0 Kit using altered reaction conditions, use the dGTP BigDye Terminator v3.0 Kit. ♦ If the sequence data still does not improve, using altered reaction conditions with the dGTP BigDye Terminator v3.0 Kit can be helpful in some cases.

Template Characteristic at or Near the Stop Region	Chemistry to Use
C-rich	BigDye terminator v3.0: Use strong denaturants such as 1 M betaine (refer to pages 7-32 and 7-33 of the *Automated DNA Sequencing Chemistry Guide* for additional methods for improving template denaturation).
Secondary structure	dGTP BigDye terminator v3.0
Certain sequence contexts or motifs **Note** In these cases, the reason for the stop may not be apparent.	dGTP BigDye terminator v3.0

The figure below shows data from a DNA clone of the HV2 region of the mitochondria D loop sequenced using the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit. This clone contains a difficult-to-sequence region, as shown in the electropherogram (Figure 1-1).

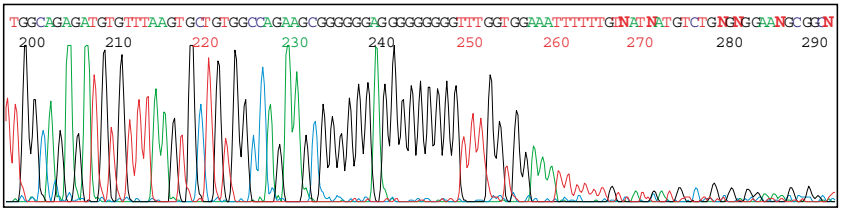


Figure 1-1 Mitochondrial DNA sequenced with BigDye terminators v3.0

Figure 1-2 below shows data from the same template, but sequenced using the dGTP BigDye terminators v3.0. Sequence beyond the stop region is obtained using this chemistry.

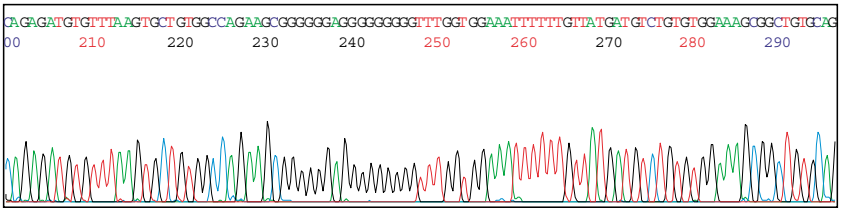


Figure 1-2 Mitochondrial DNA sequenced with dGTP BigDye terminators v3.0

Compressions

Band compressions in DNA sequencing data result from the formation of secondary structures in the DNA fragments that are not eliminated by the denaturing conditions of the gel. The fragments do not migrate according to their size, and more than one fragment can migrate at the same position. Due to the replacement of dITP by dGTP, compressions can be a problem for the dGTP BigDye Terminator v3.0 Kit.

IMPORTANT Because of band compressions, we do not recommend using the dGTP BigDye Terminator v3.0 Kit for routine sequencing. It should be used only if the standard terminator kits do not give good data.

IMPORTANT When using the ABI PRISM® 373 DNA Sequencers, compressions can be severe. Use the dGTP BigDye Terminator v3.0 Kit to sequence through the difficult regions. Then sequence the opposite strand using the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Kit with a primer that anneals to the opposite strand beyond the stop region.

Instruments

Instrument Platforms

The ABI PRISM dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit is for use with the following instruments:

- ABI PRISM® 377 DNA Sequencer (all models[1])
- ABI PRISM 373 DNA Sequencers with the ABI PRISM BigDye Filter Wheel installed[2] (Refer to the *ABI PRISM BigDye Filter Wheel User Bulletin* (P/N 4304367) for more information.)

General instructions are given for using the kit reagents to generate samples for these instruments. For more detailed instructions, refer to the appropriate instrument user's manual.

IMPORTANT This kit is not designed for use with ABI PRISM 373 DNA Sequencers and ABI PRISM® 373 DNA Sequencers with XL Upgrade that do not have the ABI PRISM BigDye Filter Wheel.

Note We are currently investigating methods to run the dGTP BigDye terminators v3.0 on capillary instruments, including the ABI PRISM® 310 Genetic Analyzer, ABI PRISM® 3700 DNA Analyzer, and the ABI PRISM® 3100 Genetic Analyzer. However, the use of this chemistry on capillary instruments is not recommended or supported by this protocol.

Thermal Cyclers

The protocols provided in this document were optimized using Applied Biosystems thermal cyclers, including:

- GeneAmp® PCR Systems 9700, 9600, and 2400
- DNA Thermal Cycler 480
- DNA Thermal Cycler (TC1)

If you use a thermal cycler not manufactured by Applied Biosystems, you may need to optimize thermal cycling conditions. Ramping time is very important. If the thermal ramping time is too fast (>1°/sec), poor (noisy) data may result.

1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.
2. Includes the ABI PRISM 373 and ABI PRISM 373 with XL Upgrade instruments.

Required Software

Dye/Filter Sets and Matrix Standards for the 377 and 373 Instruments

The dye/filter sets and matrix standards required for the 377 and 373 instruments are listed in the table below.

IMPORTANT Instrument (matrix) file for the dGTP BigDye terminators v3.0 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye primers (original).

Instrument	Dye/Filter Set	Standards for Instrument (Matrix) File Generation
377 DNA Sequencers[a]	Filter Set E	ABI PRISM® BigDye™ Matrix Standards v3.0 (P/N 4390421)
373 DNA Sequencers with the BigDye Filter Wheel[b]	Filter Set A	

a. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

b. Includes the ABI PRISM 373 and ABI PRISM 373 with XL Upgrade instruments.

Instructions for Generating Matrices

- For the 377 instruments, refer to the product insert for instructions on using the ABI PRISM BigDye Matrix Standards v3.0 (P/N 4390421) to generate matrices.
- For the 373 instruments, contact Technical Support for instructions on using the ABI PRISM BigDye Matrix Standards v3.0 (P/N 4390421) to generate matrices.

Dye Set/Primer (Mobility) Files

Available in Two Places

To analyze sequencing data generated with BigDye chemistries v3.0, you need dye set/primer (mobility) files that were created for v3.0 chemistries. The dye set/primer (mobility) files can be obtained from two places:

- The files can be installed from the two CD-ROMs or one floppy disk enclosed in the ABI PRISM BigDye Matrix Standards v3.0 (P/N 4390421).
- The files can be downloaded from the Internet.

Installing Files from the CD-ROMs or Floppy Disk Enclosed in the v3.0 Matrix Standards

If your data was collected on a...	and you wish to analyze your data using a ...	Refer to the CD-ROM or floppy disk labeled...
377 instrument	computer with the Windows NT® platform	PN 4326478, For Windows NT platform
377 or 373 instrument	Macintosh® computer with a CD-ROM drive	PN 4326479, For Macintosh platform
377 or 373 instrument	Macintosh computer with a floppy drive	PN 4326480, For Macintosh platform

Downloading Files from the Internet

Dye set/primer (mobility) files can be downloaded from our Web site:

http://www.appliedbiosystems.com/techsupp/swpps/SAsw.html

If you do not have access to the Internet, you can get the files from Applied Biosystems Technical Support, or from your local field applications specialist (call your local sales office for more information).

Reagents and Storage

Available Kit

The following kit is available:

Kit	Number of Reactions	Part Number
The ABI PRISM dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS	100	4390229

Description of Reagents

A description of the kit reagents is listed below.

- Terminator Ready Reaction Mix:
  - A-Dye Terminator
  - C-Dye Terminator
  - G-Dye Terminator
  - T-Dye Terminator
  - Deoxynucleoside triphosphates (dATP, dCTP, dGTP, dUTP)
  - AmpliTaq DNA Polymerase, FS, with thermally stable pyrophosphatase
  - $MgCl_2$
  - Tris-HCl buffer, pH 9.0
- pGEM®-3Zf(+) double-stranded DNA Control Template, 0.2 µg/µL
- –21 M13 Control Primer (forward), 0.8 pmol/µL

Storage and Use of the Kit

- Store the kit at –15 to –25 °C.
- Avoid excessive (*i.e.,* no more than 5–10) freeze-thaw cycles. Aliquot reagents in smaller amounts if necessary.
- Before each use of the kit, allow the frozen stocks to thaw at room temperature (do not heat).

IMPORTANT Mix each stock thoroughly and then centrifuge briefly to collect all the liquid at the bottom of each tube.

- Whenever possible, thawed materials should be kept on ice during use. Do not leave reagents at room temperature for extended periods.

Materials Supplied by the User

Overview

In addition to the reagents supplied in this kit, other items are required.

This section lists general materials needed for:

- Cycle sequencing
- Purifying extension products

Note Many of the items listed in this section are available from major laboratory suppliers (MLS) unless otherwise noted. Equivalent sources may be acceptable where noted.

Refer to the individual instrument protocols for the specific items needed for each instrument.

⚠ WARNING CHEMICAL HAZARD. Before handling the chemical reagents needed for cycle sequencing, read the safety warnings on the reagent bottles and in the manufacturers' Material Safety Data Sheets (MSDSs), and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Dispose of waste in accordance with all local, state/provincial, and national environmental and health regulations.

Materials for Cycle Sequencing

The table below lists the plates or tubes required for the recommended Applied Biosystems thermal cyclers (page 1-7).

Thermal Cycler	Plate or Tube	Applied Biosystems Part Number
GeneAmp PCR System 9700	MicroAmp® 96-Well Reaction Plate	N801-0560
	MicroAmp® Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp® Caps, 12 or 8/strip	N801-0534 or N801-0535
	ABI PRISM™ Optical Adhesive Cover Starter Pack or ABI PRISM® Optical Adhesive Covers	4313663 or 4311971
GeneAmp PCR System 9600	MicroAmp 96-Well Reaction Plate	N801-0560
	MicroAmp Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp Caps, 12 or 8/strip	N801-0534 N801-0535
	ABI PRISM Optical Adhesive Cover Starter Pack or ABI PRISM Optical Adhesive Covers	4313663 or 4311971
GeneAmp PCR System 2400	MicroAmp Reaction Tubes, 0.2-mL	N801-0533
	MicroAmp Caps, 12 or 8/strip	N801-0534 N801-0535
DNA Thermal Cycler 480[a]	GeneAmp® Thin-Walled Reaction Tubes, 0.5-mL	N801-0537
	GeneAmp® Thin-Walled Reaction Tubes with Flat Cap	N801-0737
DNA Thermal Cycler (TC1) [a]	GeneAmp Thin-Walled Reaction Tubes, 0.5-mL	N801-0537

a. The DNA Thermal Cycler 480 and the DNA Thermal Cycler (TC1) thermal cyclers require mineral oil that can be obtained from Applied Biosystems (P/N 0186-2302)

Materials for Purifying Extension Products

Method	Material	Supplier
Ethanol/Sodium Acetate Precipitation **Note** For 96-well reaction plates and microcentrifuge tubes.	Ethanol (EtOH), non-denatured, 95% Sodium acetate (NaOAc), 3 M, pH 4.6 Aluminum foil tape, adhesive-backed	MLS Applied Biosystems (P/N 400320) 3M (Scotch Tape P/N 431 or 439)[a]
Ethanol Precipitation **Note** For 96-well reaction plates and microcentrifuge tubes.	Ethanol (EtOH), non-denatured, 95% Aluminum foil tape, adhesive-backed	MLS 3M (Scotch Tape P/N 431 or 439)[a]
Spin Column Purification	Centri-Sep™ spin column, 1-mL, 32 columns, 100 columns Aluminum foil tape, adhesive-backed	Applied Biosystems P/N 401763, P/N 401762 3M (Scotch Tape P/N 431 or 439)[a]

a. Contact 3M in the USA at (800) 364-3577 for your local 3M representative. Use of other tapes may result in leakage or contamination of the sample.

Safety

Documentation User Attention Words

Five user attention words appear in the text of all Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below.

Note Calls attention to useful information.

IMPORTANT Indicates information that is necessary for proper instrument operation.

⚠ CAUTION Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

⚠ WARNING Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.

⚠ DANGER Indicates an imminently hazardous situation which, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

⚠ WARNING **CHEMICAL HAZARD**. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

- Read and understand the material safety data sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard Warning

⚠ WARNING CHEMICAL WASTE HAZARD. Wastes produced by Applied Biosystems instruments are potentially hazardous and can cause injury, illness, or death.

- Read and understand the material safety data sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Handle chemical wastes in a fume hood.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (*e.g.,* safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (*e.g.*, fume hood). For additional safety guidelines, consult the MSDS.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Site Preparation and Safety Guide

A site preparation and safety guide is a separate document sent to all customers who have purchased an Applied Biosystems instrument. Refer to the guide written for your instrument for information on site preparation, instrument safety, chemical safety, and waste profiles.

About MSDSs

Some of the chemicals used with this instrument may be listed as hazardous by their manufacturer. When hazards exist, warnings are prominently displayed on the labels of all chemicals.

Chemical manufacturers supply a current material safety data sheet (MSDS) before or with shipments of hazardous chemicals to new customers and with the first shipment of a hazardous chemical after an MSDS update. MSDSs provide you with the safety information you need to store, handle, transport and dispose of the chemicals safely.

We strongly recommend that you replace the appropriate MSDS in your files each time you receive a new MSDS packaged with a hazardous chemical.

⚠ WARNING CHEMICAL HAZARD. Be sure to familiarize yourself with the MSDSs before using reagents or solvents.

Ordering MSDSs

You can order free additional copies of MSDSs for chemicals manufactured or distributed by Applied Biosystems using the contact information below.

To order documents by automated telephone service:

1	From the U.S. or Canada, dial **1.800.487.6809**, or from outside the U.S. and Canada, dial **1.858.712.0317**.
2	Follow the voice instructions to order documents (for delivery by fax). **Note** There is a limit of five documents per fax request.

To order documents by telephone:

In the U.S.	Dial **1.800.345.5224**, and press **1**.
In Canada	♦ To order in English, dial **1.800.668.6913** and press **1**, then **2**, then **1** ♦ To order in French, dial **1.800.668.6913** and press **2**, then **2**, then **1**
From any other country	See the specific region under “To Contact Technical Support by Telephone or Fax (Outside North America)” .

To view, download, or order documents through the Applied Biosystems web site:

Step	Action
1	Go to **http://www.appliedbiosystems.com**
2	Click **SERVICES & SUPPORT** at the top of the page, click **Documents on Demand**, then click **MSDS.**
3	Click **MSDS Index**, search through the list for the chemical of interest to you, then click on the MSDS document number for that chemical to open a pdf of the MSDS.

For chemicals not manufactured or distributed by Applied Biosystems, call the chemical manufacturer.

Preparing the Templates

2

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Control DNA Templates

Using Control DNA

Include a control DNA template as one of the templates in a set of sequencing reactions. The results from the control can help determine whether failed reactions are the result of poor template quality or sequencing reaction failure.

Control DNA Sequence

We recommend M13mp18 as a single-stranded control and pGEM®-3Zf(+) as a double-stranded control. All Applied Biosystems DNA sequencing kits provide pGEM® control DNA. All dye terminator cycle sequencing kits include a –21 M13 forward primer for use to perform control reactions.

The partial sequence of pGEM-3Zf(+) from the –21 M13 forward primer, followed by the ensuing 1000 bases is shown in Appendix B, “Control DNA Sequence.”

An Additional Control Sold Separately

The BigDye™ terminator v3.0 sequencing standard provides an additional control to help in troubleshooting electrophoresis runs. It contains lyophilized sequencing reactions that require only resuspension and denaturation before use.

Refer to the product insert for instructions on using the sequencing standard.

Instrument	Kit	Part Number
ABI PRISM® 377 DNA Sequencers[a]	ABI PRISM® BigDye™ Terminator v3.0 Sequencing Standard	4390303
ABI PRISM® 373 DNA Sequencers with the BigDye™ Filter Wheel[b]		

a. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

b. Includes the ABI PRISM 373 and ABI PRISM 373 with XL Upgrade instruments.

Template Preparation Methods

Single- and Double-Stranded Templates

Refer to *Automated DNA Sequencing Chemistry Guide* (P/N 4305080) for information on preparing single- and double-stranded templates.

PCR Templates

Cycle sequencing provides the most reproducible results for sequencing PCR templates. Although PCR fragments can be difficult to denature with traditional sequencing methods, cycle sequencing provides several chances to denature and extend the template, which ensures adequate signal in the sequencing reaction.

Importance of Purifying Product

For optimum results, purify the PCR product before sequencing. In general, any method that removes dNTPs and primers should work. We recommend Centricon®-100 columns (P/N N930-2119). The protocol for using these columns is provided in "Purifying PCR Fragments" below.

Purifying PCR Fragments

To purify PCR fragments by ultrafiltration:

Step	Action
1	Assemble the Centricon-100 column according to the manufacturer's recommendations.
2	Load 2 mL deionized water onto the column.
3	Add the entire sample to the column.
4	Spin the column at 3000 × *g* in a fixed-angle centrifuge for 10 minutes. **Note** The manufacturer recommends a maximum speed of 1000 × *g*, but 3000 × *g* has worked well in Applied Biosystems laboratories. If you are following the manufacturer's guidelines, increase the time to compensate.
5	Remove the waste receptacle and attach the collection vial.
6	Invert the column and spin it at 270 × *g* for 2 minutes to collect the sample. This should yield approximately 40–60 µL of sample.
7	Add deionized water to bring the purified PCR fragments to the original volume.

Use of the Primer Island Transposition Kit

Overview

The dGTP BigDye terminators v3.0 are also suitable for sequencing plasmid templates generated using the Primer Island® Transposition Kit (P/N 402984). This kit uses transposons to insert primer binding sites into cloned DNA.

About Transposons

Transposons are mobile genetic elements, regions of DNA capable of inserting themselves (or copies of themselves) into the genome. Transposons encode the proteins that facilitate their insertion into the target DNA.

Inserting Artificial Transposons

This property of transposons can be exploited to place unique primer binding sites randomly throughout any large segment of DNA. These primer sites may be used subsequently as templates for PCR and/or sequencing reactions. Transposon insertion is an alternative to subcloning or primer walking when sequencing a large cloned DNA region.[1,2]

The Primer Island Transposition Kit provides reagents for generating artificial transposon insertions into target DNA *in vitro*. The artificial transposon contains the PI(+) and PI(–) priming sites. The Primer Island reagents are combined with a target DNA of choice and used to transform *Escherichia coli*.

Technique

To identify the *E. coli* carrying the transposon, the transformed bacteria are plated on Luria-Bertani (LB) agar plates containing carbenicillin and trimethoprim antibiotics. Each carbenicillin- and trimethoprim-resistant colony has integrated a copy of the transposon into the target DNA.

Follow *Primer Island Transposition Kit Protocol* (P/N 402920) for transposon insertion and template preparation.

1. Devine, S.E., and Boeke, J.D. 1994. Efficient integration of artificial transposons into plasmid targets *in vitro*: a useful tool for DNA mapping, sequencing, and functional analysis. *Nucleic Acids Res.* 22: 3765–3772.

2. Devine, S.E., Chissoe, S.L., Eby, Y., Wilson, R.K., and Boeke, J.D. 1997. A transposon-based strategy for sequencing repetitive DNA in eukaryotic genomes. *Genome Res.* 7: 551–563.

DNA Quantity

Quantitating DNA

If possible, quantitate the amount of purified DNA by measuring the absorbance at 260 nm or by some other method.

Template Quantity

The table below shows the amount of template to use in a cycle sequencing reaction.

Template	Quantity
PCR product:	
100–200 bp	1–3 ng
200–500 bp	3–10 ng
500–1000 bp	5–20 ng
1000–2000 bp	10–40 ng
>2000 bp	40–100 ng
Single-stranded	50–100 ng
Double-stranded	200–500 ng
Cosmid, BAC	0.5–1.0 µg
Bacterial genomic DNA	2–3 µg

Note In general, higher DNA quantities give higher signal intensities.

The ranges given in the table above should work for all primers. You may be able to use even less DNA, especially when sequencing with the –21 M13 primer. The amount of PCR product to use in sequencing will also depend on the length and purity of the PCR product.

Template Volume

Cycle-sequencing reactions are made up in a final volume of 20 µL. The volume allows for up to 8 µL for DNA template and 4 µL for primer (0.8 pmol/µL). If your DNA is not concentrated enough and you need to add more than 8 µL of DNA template, then you can compensate for the additional volume by using a more concentrated solution of primer.

For example, if your concentration of primers is increased from 0.8 pmol/µL to 3.2 pmol/µL, then the volume of primers can be reduced from 4 µL to 1 µL. Because less volume is used for the primers, more volume can then be added for the template. In this example, the volume of DNA template could be increased from 8 µL to 11 µL.

New Cycle Sequencing Protocols 3

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Important Protocol Changes

Changes to the Cycle Sequencing Protocol The cycle sequencing protocols used for the ABI PRISM® dGTP BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit with ABI PRISM® DNA Polymerase, FS, are changed from those used for the ABI PRISM® BigDye™ Terminator v3.0 Ready Reaction Cycle Sequencing Kit. These protocols are also different than those used in the previous version of the dGTP BigDye Terminator v3.0 kit. The cycling conditions protocols have been improved to reduce cycling time by half. They have been optimized for Applied Biosystems thermal cyclers.

Cycle Sequencing Single- and Double-Stranded DNA

Overview

This section describes how to prepare reactions and perform cycle sequencing on a variety of templates, including M13, plasmids, and PCR products.

Preparing the Reactions

The type of tube required depends on the thermal cycler that you are using. Refer to “Materials for Cycle Sequencing” on page 1-12.

To prepare the reaction mixtures:

Step	Action	
1	For each reaction, add the following reagents to a separate tube:	
	Reagent	**Quantity**
	Terminator Ready Reaction Mix	8.0 µL
	Template	See the table under “Template Quantity” on page 2-5.
	Primer	3.2 pmol
	Deionized water	q.s.
	Total Volume	20 µL
2	Mix well and spin briefly.	
3	**If using the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:**	
	Overlay the reaction mixture with 40 µL of light mineral oil.	

Cycle Sequencing on the System 9700, 9600, or 2400

To sequence single- and double-stranded DNA on the GeneAmp® PCR System 9700 (in 9600 emulation mode), 9600, or 2400:

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 10 seconds ♦ Rapid thermal ramp to 68 °C ♦ 68 °C for 2 minutes
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.
5	Proceed to Chapter 4, “Purifying Extension Products.”

a. Rapid thermal ramp is 1 °C/second.

Comparison to Original Procedure

The following changes have been made:

Original	New
Rapid thermal ramp to 96 °C	No change
96 °C for 10 seconds	No change
Rapid thermal ramp to 50 °C	Rapid thermal ramp to 68 °C
50 °C for 5 sec	Eliminated
Rapid thermal ramp to 60 °C	Eliminated
60 °C for 4 minutes	68 °C for 2 minutes

Note Two steps have been modified, and two steps have been eliminated. This is a faster procedure because of the change from a 3-step to a 2-step protocol and the use of a higher extension temperature.

Cycle Sequencing on the TC1 or 480

To sequence single- and double-stranded DNA on the DNA Thermal Cycler (TC1) or DNA Thermal Cycler 480:

Step	Action
1	Place the tubes in a thermal cycler and set the volume to 20 µL.
2	Repeat the following for 25 cycles: ♦ Rapid thermal ramp[a] to 96 °C ♦ 96 °C for 30 seconds ♦ Rapid thermal ramp to 68 °C ♦ 68 °C for 2 minutes
3	Rapid thermal ramp to 4 °C and hold until ready to purify.
4	Spin down the contents of the tubes in a microcentrifuge.
5	Proceed to Chapter 4, “Purifying Extension Products.”

a. Rapid thermal ramp is 1 °C/second.

Comparison to Original Procedure

The following changes have been made:

Original	New
Rapid thermal ramp to 96 °C	No change
96 °C for 30 seconds	No change
Rapid thermal ramp to 50 °C	Rapid thermal ramp to 68 °C
50 °C for 15 sec	Eliminated
Rapid thermal ramp to 60 °C	Eliminated
60 °C for 4 minutes	68 °C for 2 minutes

Note Two steps have been modified, and two steps have been eliminated. This is a faster procedure because of the change from a 3-step to a 2-step protocol and the use of a higher extension temperature.

Purifying Extension Products

4

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Choosing a Method of Purification

Purpose

Unincorporated dye terminators must be removed before the samples can be analyzed by electrophoresis. Excess dye terminators in sequencing reactions obscure data in the early part of the sequence and can interfere with basecalling.

Purification Methods

There are several methods you can use to purify extension products:

Purification Method	See page
Ethanol/Sodium Acetate Precipitation in 96-Well Reaction Plates	4-3
Ethanol/Sodium Acetate Precipitation in Microcentrifuge Tubes	4-6
Ethanol Precipitation in 96-Well Reaction Plates	4-9
Ethanol Precipitation in Microcentrifuge Tubes	4-11
Spin Column Purification	4-13

Spin Column vs. Precipitation

Use the method that works best for your particular application.

- Precipitation methods are cheaper and faster, but they may remove less of the unincorporated dye-labeled terminators that can obscure data at the beginning of the sequence.
- The spin column procedure removes more terminators, but is more costly and may take additional time to perform.

Ethanol/Sodium Acetate Precipitation in 96-Well Reaction Plates

Recommended Protocol

With the dGTP BigDye™ terminators v3.0, the ethanol/sodium acetate precipitation method for 96-well reaction plates produces consistent signal, while minimizing unincorporated dyes. A final 70% ethanol wash is required.

Note While this method produces the cleanest signal, it may cause loss of small molecular weight fragments.

Precipitating in 384-Well Reaction Plates

To use the ethanol/sodium acetate precipitation method for 384-well reaction plates, refer to *ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit Protocol* (P/N 4390037).

Precipitating in 96-Well Reaction Plates

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in 96-well reaction plates:

Step	Action
1	Remove the 96-well MicroAmp® reaction plate from the thermal cycler. Remove the caps from each tube.

To precipitate in 96-well reaction plates: *(continued)*

Step	Action
2	Prepare the ethanol/sodium acetate solution by combining the following for each sample: ♦ Make enough to precipitate all samples in your experiment. ♦ 3.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 62.5 µL of non-denatured 95% ethanol (EtOH) ♦ 14.5 µL of deionized water The final volume should be 80 µL for each sample. **⚠ CAUTION** **CHEMICAL HAZARD**. **3M sodium acetate** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **⚠ WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3	Add 80 µL of this ethanol/sodium acetate solution to 20 µL of reaction mixture.
4	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
5	Invert the plate a few times to mix.
6	Leave the plate at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.

To precipitate in 96-well reaction plates: *(continued)*

Step	Action
7	Place the plate in a table-top centrifuge with a tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp plate can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If this is not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
8	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the plate onto a paper towel folded to the size of the plate.
9	Place the inverted plate with the paper towel into the table-top centrifuge and spin at 50 × *g* for 1 minute.
10	Add 150 µL of 70% ethanol to each pellet.
11	Cap or seal the tubes, then invert the plate a few times to mix.
12	Spin the plate for 10 minutes at maximum speed (see step 7 above).
13	Repeat steps 8 and 9.
14	Remove the plate and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Ethanol/Sodium Acetate Precipitation in Microcentrifuge Tubes

Recommended Protocol

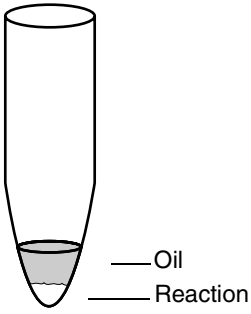
With the dGTP BigDye terminators v3.0, the ethanol/sodium acetate precipitation method in microcentrifuge tubes produces consistent signal while minimizing unincorporated dyes. A final 70% ethanol wash is required.

Note While this method produces the cleanest signal, it may cause loss of small molecular weight fragments.

Precipitating in Microcentrifuge Tubes

IMPORTANT Use non-denatured 95% ethanol rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in microcentrifuge tubes:

Step	Action
1	**IMPORTANT** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as described below. **To remove reactions run on the TC1 or DNA Thermal Cycler 480:** Place the pipette tip into the bottom of the reaction and carefully remove the reaction from the oil. Transfer as little oil as possible. 

To precipitate in microcentrifuge tubes: *(continued)*

Step	Action
2	Prepare the ethanol/sodium acetate solution by combining the following for each sample: ♦ 3.0 µL of 3 M sodium acetate (NaOAc), pH 4.6 ♦ 62.5 µL of 95% ethanol (EtOH) ♦ 14.5 µL of deionized water The final volume should be 80 µL for each sample. **⚠ CAUTION** **CHEMICAL HAZARD**. **3M sodium acetate** may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. **⚠ WARNING** **CHEMICAL HAZARD**. **Ethanol** is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3	Add 80 µL of this ethanol/sodium acetate solution to 20 µL of reaction mixture.
4	Close the tubes and vortex briefly.
5	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.
6	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If this is not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Carefully aspirate the supernatants with a separate pipette tip for each sample, then discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.

To precipitate in microcentrifuge tubes: *(continued)*

Step	Action
8	Add 250 µL of 70% ethanol to the tubes and mix briefly.
9	Place the tubes in the microcentrifuge in the same orientation as step 5 and spin for 5 minutes at maximum speed.
10	Aspirate the supernatants carefully, as in step 6.
11	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. Do not over-dry.

Ethanol Precipitation in 96-Well Reaction Plates

Unincorporated Terminators

With ethanol precipitation, residual terminator peaks may be seen. However, the recovery of small molecular weight fragments will be improved using this precipitation method.

Precipitating in 96-Well Reaction Plates

IMPORTANT Where 95% ethanol is recommended in precipitation protocols, purchase non-denatured ethanol at this concentration rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in 96-well reaction plates:

Step	Action
1	Remove the 96-well MicroAmp plate from the thermal cycler. Remove the caps from each tube.
2	Add the following for each sample: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%. **⚠ WARNING CHEMICAL HAZARD. Ethanol** is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3	Seal the tubes with strip caps or by applying a piece of 3M Scotch Tape 431 or 439 adhesive-backed aluminum foil tape. Press the foil onto the tubes to prevent any leakage.
4	Invert the plate a few times to mix.
5	Leave the plate at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.

To precipitate in 96-well reaction plates: *(continued)*

Step	Action
6	Place the plate in a table-top centrifuge with a tube-tray adaptor and spin it at the maximum speed, which must be ≥1400 × *g* but <3000 × *g*: ♦ 1400–2000 × *g*: 45 minutes ♦ 2000–3000 × *g*: 30 minutes **Note** A MicroAmp tube in a MicroAmp plate can withstand 3000 × *g* for 30 minutes. **IMPORTANT** Proceed to the next step immediately. If this is not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
7	Without disturbing the precipitates, remove the adhesive tape and discard the supernatant by inverting the plate onto a paper towel folded to the size of the plate.
8	Place the inverted plate with the paper towel into the table-top centrifuge and spin at 50 × *g* for 1 minute.
9	Add 150 µL of 70% ethanol to each pellet.
10	Cap or seal the tubes, then invert the plate a few times to mix.
11	Spin the plate for 10 minutes at maximum speed. See step 6 above.
12	Repeat steps 7 and 8.
13	Remove the plate and discard the paper towel. **Note** Pellets may or may not be visible. Vacuum drying of the samples is not necessary.

Ethanol Precipitation in Microcentrifuge Tubes

Unincorporated Terminators

With ethanol precipitation, residual terminator peaks may be seen. However, the recovery of small molecular weight fragments will be improved using this precipitation method.

Precipitating in Microcentrifuge Tubes

IMPORTANT Where 95% ethanol is recommended in precipitation protocols, purchase non-denatured ethanol at this concentration rather than absolute (100%) ethanol. Absolute ethanol absorbs water from the atmosphere, gradually decreasing its concentration. This can lead to inaccurate final concentrations of ethanol, which can affect some protocols.

To precipitate in microcentrifuge tubes:

Step	Action
1	pipette the entire contents of each extension reaction into a 1.5-mL microcentrifuge tube. **Note** If the TC1 or DNA Thermal Cycler 480 was used for thermal cycling, remove the reactions from the tubes as shown in step 1 on page 4-6.
2	Add the following for each sample: ♦ 16 µL of deionized water ♦ 64 µL of non-denatured 95% ethanol The final ethanol concentration should be 60 ± 3%. **⚠ WARNING CHEMICAL HAZARD. Ethanol** is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression and liver damage. keep away from heat, sparks, and flame. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
3	Close the tubes and vortex briefly.
4	Leave the tubes at room temperature for 15 minutes to precipitate the extension products. **Note** Precipitation times <15 minutes will result in the loss of very short extension products. Precipitation times >24 hours will increase the precipitation of unincorporated dye terminators.

To precipitate in microcentrifuge tubes: *(continued)*

Step	Action
5	Place the tubes in a microcentrifuge and mark their orientations. Spin the tubes for 20 minutes at maximum speed. **IMPORTANT** Proceed to the next step immediately. If this is not possible, then spin the tubes for 2 minutes more immediately before performing the next step.
6	Carefully aspirate the supernatants with a separate pipette tip for each sample and discard. Pellets may or may not be visible. **IMPORTANT** The supernatants must be removed completely, as unincorporated dye terminators are dissolved in them. The more residual supernatant left in the tubes, the more unincorporated dye terminators will remain in the samples.
7	Add 250 µL of 70% ethanol to the tubes and vortex them briefly.
8	Place the tubes in the microcentrifuge in the same orientation as in step 5 and spin for 10 minutes at maximum speed.
9	Aspirate the supernatants carefully, as in step 6.
10	Dry the samples in a vacuum centrifuge for 10–15 minutes or to dryness. Do not over-dry.

Spin Column Purification

Overview

This section describes the recommended spin columns for purifying extension products.

IMPORTANT Extra caution is required when dispensing samples onto the column bed. Residual dye peaks will result if samples flow through the sides of the column.

Recommended Spin Columns

We recommend CentriSep™ spin columns (Applied Biosystems, P/N 401763 for 32 columns and P/N 401762 for 100 columns).

Optimizing Spin Column Purification

IMPORTANT When using the dGTP BigDye terminators v3.0, hydrate the column for 2 hours.

Tips for optimizing spin column purification:

- Do not process more columns than you can handle conveniently at one time.
- Load the sample in the center of the column bed slowly. Make sure that the sample does not touch the sides of the column and that the pipette tip does not touch the gel surface.
- If samples are not loaded properly, peaks from unincorporated dye terminators can result.
- Spin the column at 325–730 $\times$ *g* for best results. Use the following formula to calculate the best speed for your centrifuge:

  $g = 11.18 \times r \times (\text{rpm}/1000)^2$

  where:

  g = relative centrifugal force

  r = radius of the rotor in cm

  rpm = revolutions per minute
- Do not spin for more than 2 minutes.
- Perform the entire procedure without interruption to ensure optimal results. Do not allow the column to dry out.

Performing Spin Column Purification

To perform spin column purification:

Step	Action
1	Gently tap the column to cause the gel material to settle to the bottom of the column.
2	Remove the upper end cap and add 0.8 mL of deionized water.
3	Replace the upper end cap and vortex or invert the column a few times to mix the water and gel material.
4	Allow the gel to hydrate at room temperature for at least 2 hours. **Note** Hydrated columns can be stored for a few days at 2–6 °C. Longer storage in water is not recommended. Allow columns stored at 2–6 °C to warm to room temperature before use.
5	Remove any air bubbles by inverting or tapping the column and allowing the gel to settle.
6	Remove the upper end cap first, then remove the bottom cap. Allow the column to drain completely by gravity. **Note** If flow does not begin immediately, apply gentle pressure to the column with a pipette bulb.
7	Insert the column into the wash tube provided.
8	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes to remove the interstitial fluid.
9	Remove the column from the wash tube and insert it into a sample collection tube (*e.g.*, a 1.5-mL microcentrifuge tube).
10	Remove the extension reaction mixture from its tube and load it carefully onto the center of the gel material.
11	Spin the column in a microcentrifuge at 730 × *g* for 2 minutes. **Note** If using a centrifuge with a fixed-angle rotor, place the column in the same orientation it was in for the first spin. This is important because the surface of the gel will be at an angle in the column after the first spin.
12	Discard the column. The sample is in the sample collection tube.
13	Dry the sample in a vacuum centrifuge for 10–15 minutes, or until dry. Do not over-dry.

Sample Electrophoresis

5

Chapter Summary

In This Chapter The following topics are covered in this chapter:

Before You Begin

Important Reminders

- Dye set/primer (mobility) file names for the dGTP BigDye™ terminators v3.0 are different from those for the dRhodamine terminators and BigDye terminators original and v2.0.
- If a mobility file for the wrong sequencing chemistry is used, some bases may be miscalled. This is due to different dye labeling for the different chemistries. In addition, there are differences in the mobility shifts between the dRhodamine and BigDye terminator v3.0 chemistries.
- Use the same dye set/primer (mobility) files for BigDye terminators v3.0 and dGTP BigDye terminators v3.0.

Note See “Dye Set/Primer (Mobility) Files” on page 1-9 for information on obtaining the v3.0 dye set/primer (mobility) files.

Electrophoresis on the ABI PRISM 377 DNA Sequencers

Requirements

Electrophoresis and data analysis of samples on the ABI PRISM® 377 DNA Sequencers (all models[1]) require the following:

Filter Set E Run Modules

Configuration[a]	Run Module
36-cm wtr, 1200 scans/hr, any comb	Seq Run 36E-1200
36-cm wtr, 2400 scans/hr, any comb	Seq Run 36E-2400
48-cm wtr, 1200 scans/hr, any comb	Seq Run 48E-1200

a. Any plate check and prerun module can be used on the ABI PRISM 377 DNA Sequencers.

Dye Set/Primer (Mobility) Files

Gel Formulation	Dye Set/Primer (Mobility) File
4.5% acrylamide (29:1) or 5% Long Ranger™ gel	DT377{BDv3}v1.mob

Matrix Standards

IMPORTANT Instrument (matrix) file for the dGTP BigDye terminators v3.0 and BigDye terminators v3.0 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye™ primers (original).

Dye/Filter Set	Standards for Instrument (Matrix) File Generation
E	ABI PRISM® BigDye™ Matrix Standards v3.0 (P/N 4390421)

Note Refer to the product insert for instructions on using the standards for this instrument.

1. Includes the ABI PRISM 377, ABI PRISM 377-18, ABI PRISM 377 with XL Upgrade, and the ABI PRISM 377 with 96-Lane Upgrade instruments.

Using the Lane Guide Kit

If you are using the BigDye chemistries v3.0 on the 377 instrument in conjunction with the ABI PRISM® Lane Guide™ Lane Identification Kit, refer to that kit's protocol (P/N 4313804) for instructions on resuspending and loading samples.

Using Long-Read Gel and Buffer Formulations

For longer sequencing read lengths follow the gel and buffer formulations described in user bulletin *Achieving Longer High Accuracy Reads on the 377 Sequencer* (P/N 4315153).

Resuspending and Loading the Samples

Note You can use any plate check and prerun modules.

To resuspend and load the samples:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare a loading buffer by combining the following in a 5:1 ratio (5 parts deionized formamide to 1 part EDTA with blue dextran):
♦ Deionized formamide
♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL)
⚠ WARNING CHEMICAL HAZARD. Formamide causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
⚠ CAUTION CHEMICAL HAZARD. EDTA may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.</td></tr>
<tr><td>2</td><td>Resuspend each sample pellet in loading buffer as follows:
<table>
<tr><th>Template</th><th>Volume (µL):
18- or 36-well</th><th>Volume (µL):
48-, 64-, or 96-well</th></tr>
<tr><td>PCR product, plasmid, M13</td><td>6–8</td><td>4–6</td></tr>
</table></td></tr>
<tr><td>3</td><td>Vortex and spin the samples.</td></tr>
<tr><td>4</td><td>Heat the samples at 95 °C for 2 minutes to denature. Place on ice until ready to load.</td></tr>
</table>

To resuspend and load the samples: *(continued)*

Step	Action		
5	Load each sample into a separate lane of the gel as follows:		
	Template	**Volume (µL): 18- or 36-well**	**Volume (µL): 48-, 64-, or 96-well**
	PCR product, plasmid, M13	0.75–2.0	0.5–1.5

Note If a weak signal is obtained on the ABI PRISM 377 DNA Sequencer with XL Upgrade, rerun the samples using a CCD gain of 4. Refer to the ABI PRISM 377 *DNA Sequencer XL Upgrade User's Manual* (P/N 904412) for more information.

Electrophoresis on the 373 Instrument with BigDye Filter Wheel

Requirements

General guidelines are provided below for running the ABI PRISM® 373 DNA Sequencers with the ABI PRISM® BigDye™ Filter Wheel[2] installed. For more detailed instructions, please refer the user's manual for your 373 instrument or to user bulletin *Using the ABI PRISM 373 BigDye Filter Wheel* (P/N 4304367).

Gel

For 48-cm well-to-read (wtr), we recommended 5% Long Ranger™ gel.

New Dye Set/Primer (Mobility) Files

Gel Formulation	Dye Set/Primer (Mobility) File
5% Long Ranger gel, 48-cm wtr[a]	DT373{BDv3}v1.mob

a. If you are running other wtr lengths, these are currently being tested. Please contact Technical Support.

Matrix Standards

IMPORTANT Instrument (matrix) file for the dGTP BigDye terminators v3.0 cannot be used for the BigDye terminators (original), BigDye terminators v2.0, dRhodamine terminators, or BigDye primers (original).

Dye/Filter Set	Standards for Instrument (Matrix) File Generation
A (For use with the BigDye Filter Wheel)	ABI PRISM® BigDye™ Matrix Standards v3.0 (P/N 4390421)

Note For instructions on using the matrix standards (P/N 4390421) for the 373 instruments, contact Technical Support.

2. Includes the ABI PRISM 373 and ABI PRISM 373 with XL Upgrade instruments.

Resuspending and Loading the Samples

To resuspend and load the samples:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Prepare a loading buffer by combining the following in a 5:1 ratio (5 parts deionized formamide to 1 part EDTA with blue dextran):
♦ Deionized formamide
♦ 25 mM EDTA (pH 8.0) with blue dextran (50 mg/mL)

⚠ WARNING CHEMICAL HAZARD. Formamide causes eye, skin, and respiratory tract irritation. It is a possible reproductive and birth defect hazard. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

⚠ CAUTION CHEMICAL HAZARD. EDTA may cause eye, skin, and respiratory tract irritation. Please read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.</td></tr>
<tr><td>2</td><td>Resuspend each sample pellet in loading buffer as follows:
<table>
<tr><th rowspan="2">Template</th><th colspan="4">Volume (µL)</th></tr>
<tr><th>18 or 24 well</th><th>32 or 36 well</th><th>48-well</th><th>64-well</th></tr>
<tr><td>PCR product, plasmid, M13</td><td>3–4</td><td>3–4</td><td>2–4</td><td>2–4</td></tr>
</table></td></tr>
<tr><td>3</td><td>Vortex and spin the samples.</td></tr>
<tr><td>4</td><td>Heat the samples at 95 °C for 2 minutes to denature. Place on ice until ready to load.</td></tr>
<tr><td>5</td><td>Load each sample into a separate lane of the gel as follows:
<table>
<tr><th rowspan="2">Template</th><th colspan="4">Volume (µL)</th></tr>
<tr><th>18 or 24 well</th><th>32 or 36 well</th><th>48-well</th><th>64-well</th></tr>
<tr><td>PCR product, plasmid, M13</td><td>3–4</td><td>3–4</td><td>2–2.5</td><td>2</td></tr>
</table></td></tr>
</table>

Selecting Sequencing Primers

A

Selecting Sequencing Primers

Overview

The choice of sequencing primer sequence, method of primer synthesis, and approach to primer purification can have a significant effect on the quality of the sequencing data obtained in dye terminator cycle sequencing reactions with this kit.

These decisions are particularly important when sequencing is done on real-time detection systems where signal strength is critical. Some of the recommendations given here are based on information that is general knowledge, while others are based on practical experience gained by Applied Biosystems scientists.

Recommendations

The following recommendations are provided to help optimize primer selection:

- Primers should be at least 18 bases long to ensure good hybridization.
- Avoid runs of an identical nucleotide. This is especially true for guanine, where runs of four or more Gs should be avoided.
- Keep the G-C content in the range 30–80%.
- For cycle sequencing, primers with melting temperatures (T_m) above 45 °C produce better results than primers with lower T_m.
- For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the T_m>45 °C.
- Use of primers longer than 18 bases also minimizes the chance of having a secondary hybridization site on the target DNA.

- Avoid primers that have secondary structure or that can hybridize to form dimers.
- Several computer programs for primer selection are available. They can be useful in identifying potential secondary structure problems and determining if a secondary hybridization site exists on the target DNA.

Control DNA Sequence

B

Control Sequence

Partial Sequence of pGEM-3Zf(+)

The pGEM®-3Zf(+) sequence below is the sequence of the –21 M13 forward primer, followed by the ensuing 1000 bases.

TGTAAAACGACGGCCAGT (–21 M13 primer)

GAATTGTAAT	ACGACTCACT	ATAGGGCGAA	TTCGAGCTCG	40
GTACCCGGGG	ATCCTCTAGA	GTCGACCTGC	AGGCATGCAA	80
GCTTGAGTAT	TCTATAGTGT	CACCTAAATA	GCTTGGCGTA	120
ATCATGGTCA	TAGCTGTTTC	CTGTGTGAAA	TTGTTATCCG	160
CTCACAATTC	CACACAACAT	ACGAGCCGGA	AGCATAAAGT	200
GTAAAGCCTG	GGGTGCCTAA	TGAGTGAGCT	AACTCACATT	240
AATTGCGTTG	CGCTCACTGC	CCGCTTTCCA	GTCGGGAAAC	280
CTGTCGTGCC	AGCTGCATTA	ATGAATCGGC	CAACGCGCGG	320
GGAGAGGCGG	TTTGCGTATT	GGGCGCTCTT	CCGCTTCCTC	360
GCTCACTGAC	TCGCTGCGCT	CGGTCGTTCG	GCTGCGGCGA	400
GCGGTATCAG	CTCACTCAAA	GGCGGTAATA	CGGTTATCCA	440
CAGAATCAGG	GGATAACGCA	GGAAAGAACA	TGTGAGCAAA	480
AGGCCAGCAA	AAGGCCAGGA	ACCGTAAAAA	GGCCGCGTTG	520
CTGGCGTTTT	TCCATAGGCT	CCGCCCCCCT	GACGAGCATC	560
ACAAAAATCG	ACGCTCAAGT	CAGAGGTGGC	GAAACCCGAC	600
AGGACTATAA	AGATACCAGG	CGTTTCCCCC	TGGAAGCTCC	640
CTCGTGCGCT	CTCCTGTTCC	GACCCTGCCG	CTTACCGGAT	680
ACCTGTCCGC	CTTTCTCCCT	TCGGGAAGCG	TGGCGCTTTC	720
TCATAGCTCA	CGCTGTAGGT	ATCTCAGTTC	GGTGTAGGTC	760
GTTCGCTCCA	AGCTGGGCTG	TGTGCACGAA	CCCCCCGTTC	800
AGCCCGACCG	CTGCGCCTTA	TCCGGTAACT	ATCGTCTTGA	840
GTCCAACCCG	GTAAGACACG	ACTTATCGCC	ACTGGCAGCA	880
GCCACTGGTA	ACAGGATTAG	CAGAGCGAGG	TATGTAGGCG	920
GTGCTACAGA	GTTCTTGAAG	TGGTGGCCTA	ACTACGGCTA	960
CACTAGAAGG	ACAGTATTTG	GTATCTGCGC	TCTGCTGAAG	1000

C

Technical Support

Services & Support

Applied Biosystems Web Site

To access the Applied Biosystems Web site, go to:

http://www.appliedbiosystems.com

At the Applied Biosystems Web site, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Applied Biosystems Web site provides a list of telephone and fax numbers that can be used to contact Technical Support.

Index

T

Applied Biosystems
850 Lincoln Centre Drive
Foster City, California 94404-1128
USA

P/N 4390038 Rev. D